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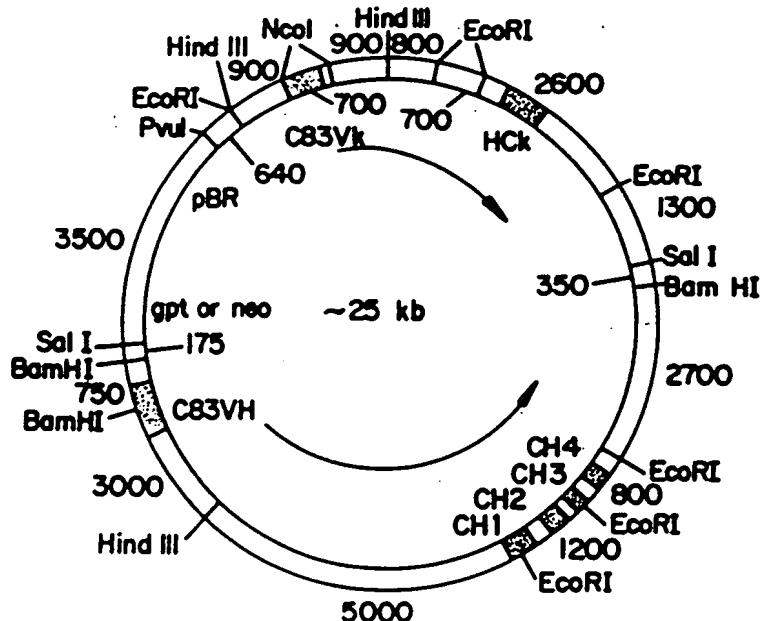
(57) Abstract

Human monoclonal antibodies are expressed by recombinant mammalian cells transformed with vectors containing genes encoding the heavy and light chains of the human monoclonal antibodies. The genes were isolated from a genomic library constructed from a human/human or human/murine hybridoma which produces the antibodies.

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DESIGNATIONS OF "DE"

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Method for Improving Human Monoclonal Antibody Production

Background

The successful utilization of human monoclonal
05   antibodies for treatment and diagnosis of human
disease is potentially limited by difficulties
associated with their commercial production.
Although murine monoclonal antibodies are relatively
easy to generate in commercial quantities, they have
10   been associated with immunogenicity in humans which
reduces their effectiveness in vivo. Development of
an immune response against mouse proteins can
neutralize the therapeutic effects of murine
monoclonal antibodies and trigger potentially
15   dangerous anaphylactic reactions.

Human monoclonal antibodies offer several
advantages over murine monoclonal antibodies,
particularly as potential pharmaceutical agents.
For example, human monoclonal antibodies are not
20   likely to provoke an immune response comparable to
the human anti-mouse antibody (HAMA) response
typically seen after administration of murine
antibodies. A HAMA response may accelerate
clearance of a circulating monoclonal antibody and
25   block its binding to an antigen thereby reducing its
effectiveness. Human monoclonal antibodies may be
more effective in interacting with the human immune
system to activate therapeutically useful effector
functions, such as antibody-dependent cellular
30   cytotoxicity, phagocytosis or complement fixation.

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Human antibodies also represent a different
repertoire of specificities, and therefore may
recognize epitopes not detected by foreign
antibodies. The effectiveness of human antibodies
05 is less likely to be compromised by problems
associated with immunogenicity such as immune
complex formation or anaphylaxis.

In spite of the many advantages of human
monoclonal antibodies for therapy, their potential
10 has not been realized, primarily because technical
obstacles have limited the number and quality of
cell lines that secrete them. Human hybridoma
technology suffers from a number of technical
shortcomings. Hybrids formed by fusion of mouse
15 myeloma cells with human lymphoid cells display a
preferential loss of human chromosomes making it
difficult to achieve long-term production of human
monoclonal antibodies. The use of human myeloma
cell lines as fusion partners has been successful in
20 some cases but there remains a problem with long
term stability. Human monoclonal antibodies are
difficult to generate and produce in commercial
quantities because of these problems. Antibodies
that have been generated, either from totally human
25 hybridomas or human-rodent heterohybridomas can be
produced only at low levels. These hybridomas often
suffer from low antibody production and instability
of the antibody-producing phenotype, presumably due
to loss of crucial chromosomes.
30   Clinical immunology research and in vivo
therapy would benefit greatly if methods were

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available for reproducibly generating human
monoclonal antibodies.

Summary of the Invention

     The present invention provides a method for
05 producing selected human monoclonal antibodies using
recombinant DNA technology. In this method, regions
of human immunoglobulin genes, generally derived
from a hybridoma, are cloned and expressed in a host
cell. The method involves preparing a first nucleic
10 acid sequence which encodes a human heavy chain of
the selected monoclonal antibody and a human
regulatory sequence, and a second nucleic acid
sequence which encodes a human light chain of the
selected monoclonal antibody and a human regulatory
15 sequence. The first and second nucleic acid
sequences are used to transform a host cell. The
transformed host cells are cultured under conditions
appropriate to selectively grow the transformed
cells. Recombinant monoclonal antibodies produced
20 by the transformed host cell are then recovered.

     The recombinant monoclonal antibodies produced
by the method are substantially identical to the
original human monoclonal antibodies, i.e., the
monoclonal antibodies produced by the hybridoma.
25 Transformed host cells produced by the present
method are stable over many generations, and produce
higher quantities of the human monoclonal antibody
than the original hybridomas.

     The present method has several advantages,
30 including improved production levels of the

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monoclonal antibody and stability of the transformed
host cells. The antibody genes are stably
integrated into the host cell genome and can be
maintained in the transformed host cell, for
05 example, by appropriate drug selection, resulting in
a high level of expression of recombinant human
monoclonal antibodies. The present method allows
the monoclonal antibody isotype to be selected on a
rational instead of a random basis. Thus, the
10 isotype of the human monoclonal antibody can be
chosen to maximize therapeutic effectiveness.

Brief Description of the Figures
        Figure 1 is a schematic representation of the
DNA probes J_H and J_K used to screen the HA-1A
15 library.
        Figure 2 is a schematic representation of
plasmids pSV2neoHuk(S) and pSV2gptHuk(S).
        Figure 3 is a graph showing the results of a
lipid A binding assay which shows that binding of
20 the monoclonal antibody produced by the 148 cell
line is indistinguishable from the binding of the
original HA-1A C83 antibody.
        Figures 4A and 4B are graphs showing the
results of experiments comparing the C83 cell line
25 with the 148 cell line for antibody production (4A)
and cell number (4B).
        Figure 5 is a schematic representation of
plasmids pSV2gptC83DP and pSV2neoC83DP.

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<u>Detailed Description of the Invention</u>

        In the method of the invention, nucleic acid
sequences, or genes, are prepared which encode human
heavy and light chains of a selected monoclonal
05 antibody, and human regulatory nucleotide sequences
specific for the genes. Alternatively, the first
and second nucleic acid sequences can encode just
the variable regions of the heavy and light chains.
The first nucleic acid sequence encodes all or a
10 portion or the heavy chain and its associated
regulatory sequence, and the second nucleic acid
sequence encodes all or a portion of the light chain
and its associated regulatory sequence. The genes
are generally derived from a hybridoma which
15 produces the selected monoclonal antibody but can be
obtained from other sources which contain the
nucleotide sequences encoding the antibody proteins.
The genes can be derived from different hybridomas,
that is, the light chain sequence from one hybridoma
20 and the heavy chain sequence from a different
hybridoma. The hybridoma can be any human/human or
human/murine hybridoma of choice which produces the
selected human monoclonal antibody.

        As used herein, the term "human/human
25 hybridoma" means fused hybrid cell lines created by
fusing a B lymphocyte with a long-lived neoplastic
plasma cell, or a T lymphocyte, with a lymphoma
cell, wherein the fusion partners of both are of
human origin. A "human/ murine hybridoma" refers to
30 fused hybrid cell lines wherein one of the fusion
partners is of murine origin.

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The expression "nucleic acid sequence" or
"nucleotide sequence" refers to a linear segment or
polymer of deoxyribonucleic acid (DNA) or
ribonucleic acid (RNA). The polymer of DNA or RNA
05 can be single- or double-stranded, optionally
containing synthetic, non-natural, or altered
nucleotides capable of incorporation into DNA or RNA
polymers.

The first and second nucleotide sequences
10 preferably include the regulatory nucleotide se-
quences which control the transcription and trans-
lation of the genes. The terms "human regulatory
nucleotide sequence" or "human regulatory sequence",
as used herein, refer to a nucleotide sequence of
15 human origin which is located 5' and/or 3' to a
nucleotide sequence the transcription of which is
controlled by the human regulatory nucleotide
sequence in conjunction with the protein synthetic
apparatus of the cell. A human regulatory nucleo-
20 tide sequence can include a promoter region, as that
term is conventionally employed by those skilled in
the art. A promoter region can include, for
example, an association region recognized by an RNA
polymerase, one or more regions which control the
25 effectiveness of transcription initiation in
response to physiological conditions, and the
transcription initiation sequence and the regulatory
elements required for expression of the gene (i.e.,
promoter, enhancer, octamer, splicing and
30 polyadenylation signals).

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In one embodiment of the present method, nucleic acid sequences encoding the intact heavy chains and light chains of the selected monoclonal antibody are isolated from a hybridoma by
05 constructing a genomic library from the hybridoma DNA. The genomic library is constructed by well-known techniques, e.g., utilizing a bacteriophage vector, such as lambda (λ) gt10 or λ EMBL-3. The vector is then used to infect a host cell, wherein
10 the insert DNA is amplified. In a preferred embodiment of the present method, a genomic library is prepared from the hybridoma DNA and cloned into the bacteriophage vector λ EMBL-3.

The genomic library is screened or probed to
15 determine which segments of the DNA in the library encode the heavy and light chains of the antibody. Heavy and light chain DNA probes are used to screen the genomic library. For example, DNA probes derived from the human light and heavy chain J
20 regions of the immunoglobulin loci (hereinafter, J_H and J_K for heavy and light chains, respectively) can be used. Other probes from the immunoglobulin loci can be used, for example, constant region sequences from the heavy and light chain genes. Screening of
25 the DNA library can be performed as described by Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY (1982).

DNA clones which have been identified as containing the desired human heavy and light chain
30 genes are isolated from the genomic library. Restriction endonuclease maps of the clones are

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constructed and compared to published maps to verify
that they are derived from the immunoglobulin loci.
Ravetch et al., Cell, 27:583-591 (1981); Klobeck and
Zachaw, Nucleic Acids Research, 14:4591-4603 (1986).
05 The variable regions can be isolated and used as
probes in Northern blot analysis (Maniatis et al.,
supra.) to verify that these sequences are expressed
in the original hybridoma.

The isolated nucleic acid sequences are sub-
10 cloned by standard techniques into vectors, e.g.,
plasmid or viral vectors. Intact heavy and light
chain genes can be cloned into separate plasmids or
combined into a single large plasmid. If a
different isotype is desired, or if only the
15 variable region is cloned for the heavy or light
chain, the variable region alone is cloned into
vectors containing previously cloned constant
regions.

The regulatory elements required for expression
20 of the antibody genes in the host cell (i.e.,
promotor, enhancer, octamer, splicing and
polyadenylation signals) are generally present on
the cloned DNA, as part of the regulatory sequences.
In addition to the nucleic acid sequences encoding
25 the heavy and light chains and associated regulatory
sequences, the vector can also include a marker gene
for selection in mammalian cells, which allows the
host cells expressing the foreign DNA to grow in
selective media. For example, the plasmid vectors
30 pSV2gpt (ATCC No. 37145) or pSV2neo (ATCC No. 37149)
are particulary useful in the present method.

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Plasmid pSV2gpt contains a gene encoding a protein
necessary to confer resistance to the drug
mycophenolic acid, and pSV2neo contains a gene
encoding a protein necessary to confer resistance to
05 the drug G418. To allow growth in bacterial cells
for ease of manipulation, an antibiotic resistance
gene such as Amp^r (ampicillin resistance) can be
included in the vector. Plasmids which are
particularly useful are plasmids containing the
10 human CK regions. Oi and Morrison, Biotechniques,
4:214-221 (1986).

The vectors containing the cloned antibody
genes are transfected into a suitable host cell line
by any known transfection method, e.g., electropora-
15 tion, calcium phosphate precipitation, DEAE-dextran,
or protoplast fusion. Any mammalian cell that
supports high levels of synthesis and secretion of
functional antibody molecules can be used as a host
cell. A well characterized, non-producing murine
20 myeloma cell line is particularly useful as a host.
A preferred host cell line is a murine myeloma cell
line which lacks any non-characterized human DNA,
viral nucleic acids or viral particles. Utilizing a
murine myeloma host cell reduces or eliminates the
25 possibility of contamination of the human monoclonal
antibody preparation with human viruses or
uncharacterized human DNA, which could occur if a
human host cell was used. Examples of suitable
murine myeloma host cells include SP2/0-Ag14 and
30 P3X63-Ag8.653, both of which are available from the
American Type Culture Collection.

After transfection, the cells are grown in
selective media such that only cells that have
acquired the transfected DNA can survive. As

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previously stated, the plasmid vector contains a
selectable marker gene encoding a protein which
confers resistance to certain drugs on transformed
cells containing the gene. Suitable selective drugs
05 include G418, mycophenolic acid and hygromycin.
        Cell clones that express or secrete antibody
are identified, for example, by assaying the culture
supernatant for the presence of antibody by any
conventional assay technique, such as enzyme-linked
10 immunosorbent assay (ELISA) or particle con-
centration fluorescence immunoassay. Monoclonal
antibodies produced by the transformed cell line can
be purified by conventional techniques, such as
protein A affinity chromatography or standard
15 biochemical procedures.
        In one embodiment of the present invention, a
library of genomic DNA fragments was constructed
from the DNA of a human-derived hybridoma which
produces a human monoclonal antibody specific for
20 the lipid A portion of a bacterial gram-negative
lipopolysaccharide. The genomic library was
constructed in a bacteriophage vector, λ EMBL-3,
using standard recombinant DNA techniques. The
library was screened with a J region probe from the
25 human kappa (κ) locus and a J region probe from the
human heavy chain locus. These probes hybridized
with the rearranged human light and heavy chain
genes. Positive clones containing the variable
regions of the heavy and light chains were isolated
30 and characterized by restriction enzyme mapping.

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The light chain variable region was cloned into
a plasmid vector already containing the human κ
region. The entire heavy chain gene, including
variable and μ constant regions, was cloned into the
05 resulting plasmid, so that functional antibody genes
were recreated. Two such plasmids were constructed
differing only in the selectable marker which was
employed (i.e., gpt and neo) to ensure the presence
of at least two copies of each gene (i.e., heavy and
10 light chain genes) in the cell line.

The plasmids were transfected into a host
murine cell line, SP2/0, by electroporation. The
SP2/0 cell line was chosen to maximize antibody
production and to minimize potential virus
15 contamination of the product. This cell line grows
well and has previously been used to express
chimeric mouse-human antibodies. The cells were
cultured in media containing drugs which selectively
allow growth of cells expressing the drug-resistance
20 genes, that is, cells which have incorporated and
express the plasmid DNA. Drug-resistant clones were
tested for the presence of secreted antibody in the
cell supernatant. The clones producing the highest
level of antibody were then subcloned, and after
25 several subcloning cycles, a stable, high-level
producer was selected.

The transfected cells were stable over many
generations because the human immunoglobulin (Ig)
genes become integrated into the host cell genome.
30 Drug selection can be maintained, but is not always
necessary, to ensure stability of the transformed

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cell lines. Some subclones maintain a high level of
antibody production in the absence of drug
selection.

The resulting antibody was then analyzed in the
05   a lipid A binding assay to demonstrate its
equivalence with the original human monoclonal
antibody derived from the hybridoma. The binding
activity of the recombinant human monoclonal anti-
bodies produced by the transformed cells was in-
10   distinguishable from the binding activity of anti-
bodies produced by the original hybridoma.

Monoclonal antibodies produced by the present
method can be used for in vitro, clinical and/or
diagnostic purposes, such as immunoassays. The
15   present monoclonal antibodies are particularly
useful for in vivo therapeutic purposes due to the
increased quantities of uniform antibodies which are
produced by the present method. For in vivo
purposes, human monoclonal antibodies are superior
20   to mouse immunoglobulins as they generally persist
in the circulation for longer periods, and do not
elicit a strong antibody response in the human. The
present human recombinant monoclonal antibodies can
be complexed with a drug or a cytotoxin or labeled
25   with a radionuclide for therapeutic applications or
a radionuclide for in vivo imaging of tumors, for
example. Other applications include use in
vaccines, or active immunization, e.g., with
antiidiotypic antibodies to raise antibodies against
30   pathogens not suitable for conventional vaccines and

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modulation of autoimmune and/or endocrine conditions
with antireceptor antibodies.

The present invention is further illustrated by
the following Exemplification.

05 EXEMPLIFICATION

Materials and Methods

Cell Lines

HA-1A (cell line C83:MCB; M83-19, Centocor,
Inc., Malvern, PA) is a mouse-human hybridoma
10 generated by fusion of an EBV-transformed human
splenic B-lymphocyte with a mouse-human hetero-
myeloma. HA-1A secretes a human IgM,K antibody
(referred to herein as "HA-1A antibody" or "HA-1A
C83") with immunoreactivity towards the lipid A
15 portion of bacterial gram-negative lipopoly-
saccharide. Growth medium for HA-1A was Iscove's
medium supplemented with 5% fetal bovine serum
(FBS). SP2/0 cells were obtained from the American
Type Culture Collection (ATCC #1581) and grown in
20 Iscoves medium supplemented with 10% FBS.

Hybridization Probes

The DNA probes for screening the HA-1A library
are shown schematically in Figure 1. The human
heavy chain J_H probe is a 6.0 kb BamHI-HindIII
25 fragment derived from the heavy chain locus
containing all four J_H exons. The human light chain
probe is a 1.8 kb SacI fragment from the kappa locus

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containing the five J_K exons. ^{32}P-labeled probes
were prepared by nick translation using a kit
obtained from Amersham, Inc. (Arlington Heights,
IL). Unincorporated nucleotides were removed by
05 centrifugation through a Sephadex G-50 column. The
specific activities of the probes were 5-10 x 10^8
cpm/μg.

Construction and Screening of a Genomic Expression
Library

10   A genomic DNA library was constructed from
HA-1A DNA from the cell line C83:MCB; M83-19
(hereinafter "C83"), a mouse/human heterohybridoma
which produces a human anti-lipid A IgM monoclonal
antibody. HA-1A genomic DNA was partially digested
15 with restriction endonuclease Sau 3A and the
resulting fragments were size-fractionated on a
10-40% sucrose density gradient. DNA fragments of
approximately 15-23 kilobases (kb) were ligated to
bacteriophage λ EMBL-3 arms previously digested with
20 BamHI, and the resulting DNA was packaged in vitro
into phage particles using Packagene (Promega
Biotech, Inc., Madison, WI) according to the
manufacturer's instructions. The phage packaged DNA
was plated on 150 mm agar plates at a density of
25 30,000 plaques per plate.
        The library was screened with the heavy chain
and light chain J region ^{32}P-labeled probes
according to the method described by Maniatis et
al., supra. Plaque hybridizations were carried out
30 in 5X SSC, 50% formamide, 2X Denhardt's reagent, 200

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μg/ml denatured salmon sperm DNA at 42°C for 18-20
hours. Final washes were in 0.5X SSC, 0.1% SDS at
65°C. Positive clones were identified after
autoradiography and isolated after at least three
05 rounds of plaque purification.

DNA Sequencing
        The clones were characterized by restriction
mapping using standard methods as described by
Maniatis et al., supra. Restriction endonuclease
10 maps were determined for HA-1A V_H and V_K clones.
Appropriate DNA fragments were subcloned into MP18
and MP19 and sequenced using the dideoxy method,
with Sequenase (U.S. Biochemical Corp., Cleveland,
OH) according to the manufacturer's instructions.
15 The amino acid translations were compared with
previously sequenced human light and heavy chains.
A Northern blot analysis showed that the cloned V
regions hybridized with the appropriate size RNAs
from the original C83 cell line, demonstrating that
20 the cloned sequences were expressed in C83.

DNA Transfection using Electroporation
        The light chain V region was then cloned into a
plasmid expression vectors containing the human CK
region and selectable markers, gpt, which confers
25 resistance to the drug mycophenolic acid, and neo,
which confers resistance to the drug G418. The
starting plasmids, pSV2gptHCK (s) and pSV2neoHCK
(s), are shown schematically in Figure 2. These
plasmids were prepared from plasmids pSV2gpt (ATCC
30 No. 37145) and pSV2neo (ATCC No. 37149) and a human
CK region. The human CK regions were obtained from
Sherie Morrison and are described by Oi and Morrison
in Biotechniques, 4:214-221 (1986).

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A second clone was isolated that contained an
entire rearranged human heavy chain gene of the IgM
type. Restriction enzyme mapping and DNA sequencing
confirmed its similarity to other human antibody
05 sequences. In addition, the variable region (V) se-
quence was shown to be expressed in the C83 cell
line by Northern blot analysis. This gene was
subcloned into the expression vectors already con-
taining the light chain gene segments, resulting in
10 two plasmids, pSV2gptC83DP and pSV2neoC83DP, with
the potential for expressing both heavy and light
chain genes derived from C83. A schematic
representation of the plasmids is shown in Figure 5.

Plasmid DNA to be transfected was purified by
15 centrifugation to equilibrium in ethidium
bromide/cesium chloride gradients two times. 10-50
μg of plasmid DNA was added to 10^7 SP2/0 cells in
phosphate buffered saline (PBS) on ice, and the
mixture was placed in a Biorad electroporation
20 apparatus. (BioRad Laboratories, Richmond, CA)
Electroporation was performed at 200 volts and the
cells were plated out in 96 well microtiter plates.
Drug selection was applied after 48 hours and drug
resistant colonies were identified after 1-2 weeks.
25 Selective medium included 0.25 μg/ml mycophenolic
acid, 1.25 μg/ml hypoxanthine, 25 μg/ml xanthine,
and 0.5 mg/ml G418.

Quantitation of Antibody Production
Tissue culture supernatant was analyzed for
30 human IgM protein content by ELISA assay using goat

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anti-human IgM Fc5u antibody (Jackson Laboratories,
Avondale, PA). Human IgM (Jackson Laboratories) was
used to generate a standard curve.

Partial_Purification_of_Recombinant_HA-1A_Antibodies
05   The recombinant antibody was purified by
addition of 4% (w/v) polyethylene glycol (MW 6000)
to clarified cell supernatant. After gentle mixing
overnight at 4°C, the precipitate was collected by
low speed centrifugation and solubilized in 0.05 M
10 Tris/0.2 M NaCl/0.1M glycine, pH 8.6. The sample
was then dialyzed against 0.3 M NaCl, 0.01 M sodium
phosphate, pH 7.2.

Lipid_A_Binding_Assay
       Lipid A binding activity was determined using a
15 solid phase ELISA assay with monophosphoryl lipid A
from S._minnesota R595 (RIBI Immunochem Research,
Inc., Hamilton, MT) coated on microtiter plates and
an alkaline phosphatase-conjugated goat anti-human
IgM antibody (Jackson Laboratories).

20 RESULTS

Cloning_of_the_HA-1A_Heavy_and_Light_Chain_Genes
       Several positive clones were isolated from an
HA-1A genomic λ EMBL-3 library using either heavy or
light chain probes. Following at least three rounds
25 of plaque purification, bacteriophage DNA was
isolated, digested with various restriction enzymes,
and fractionated on 1% agarose gels to generate

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physical maps of the clones. The DNA was
transferred to nitrocellulose and the blots were
hybridized with J_H or J_K ^{32}P-labeled probes to
determine which fragments contained the adjacent
05 variable regions. For the light chain, a 2.5 kb
HindIII fragment was identified that hybridized to
the J_K probe. This fragment was used as a
^{32}P-labeled probe to verify that the V region
sequence was expressed as RNA in the HA-1A hybridoma
10 by Northern analysis. The 2.5 kb HindIII fragment
was subsequently used for expression of the HA-1A
light chain variable region. Restriction enzyme
mapping indicated a heavy chain clone contained an
entire rearranged μ heavy chain gene. This region
15 was excised from the λ EMBL-3 phage using flanking
SalI sites in the vector, and the 14 kb SalI
fragment was used to express the HA-1A heavy chain.

Sequence Analysis of HA-1A Variable Regions
       The nucleotide sequences of the HA-1A antibody
20 heavy and light chain variable regions were
determined along with the deduced amino acid
sequences, and were compared to the nucleotide
sequences of the heavy and light chain variable
regions of the recombinant 148 antibody. Comparison
25 of the deduced HA-1A light chain sequence with human
and mouse variable region sequence indicated that
the light chain of HA-1A is a member of the human V_k
IIIb subgroup, and is the result of joining of the V
regions to the J_{K5} exon. The HA-1A heavy chain
30 sequence reveals that it is a member of the human

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V_HII subgroup, and utilizes the human J6 exon. The
deduced amino acid sequences of the 148 antibody are
clearly human in character, and the first 95 amino
acids of the light chain are identical to the
05 sequence of a human IgM antibody, and the first 20
residues of the N-terminal amino acid sequences
HA-1A antibody are identical to the seqeunce encoded
in the recombinant light chain DNA.

Construction of Expression Plasmids

10   The 2.5 kb HindIII fragment containing the
putative HA-1A light chain variable region was
cloned into the HindIII sites of plasmids
pSV2gptHuk(S) and pSV2neoHuk(S), respectively.
These plasmids contain the human CK region and
15 dominant selectable markers gpt and neo, for
selection in mammalian cells (Figure 2). The 14 kb
SalI fragment containing the complete putative HA-1A
heavy chain gene was cloned into the Sal I sites of
the vectors containing the light chain variable
20 region. The resulting plasmids, pSV2gpt283DP and
pSV2neoC83DP, (shown schematically in Figure 5),
differ only in the selectable marker employed, and
contain single copies of both heavy and light chain
genes. The expression plasmids were designed to
25 direct expression of the antibody genes using the
natural cis-acting regulatory sequences linked to
the genes including the octamer sequences,
promoters, enhancers, splice signals, and poly A
addition signals.

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     To express the recombinant antibody, the two
plasmids were cotransfected into the nonproducing
murine myeloma-derived cell line SP2/0 and a double
selection with mycophenolic acid and G418 was used
05 to obtain stable transfectants. Two copies of each
gene were transfected to augment the copy number in
recipient cells, and to increase the likelihood of
favorable integration sites. Resistant colonies
were expanded and subclones were generated from the
10 clones secreting the highest level of human IgM
antibody as measured by ELISA assay. One subclone,
designated cell line C83-148-3F3-14-21-31
(hereinafter, the "148" cell line or "C83/148"), was
chosen for further study.

15 <u>Characterization of 148 Recombinant Antibody</u>
     HA-1A antibody is a pentameric IgM molecule.
Proper assembly and secretion of pentameric IgM
encoded by transfected genes requires elements not
supplied by the heavy and light chain gene
20 constructs themselves. To obtain pentameric
recombinant IgM, such elements must be present in
the recipient cell line. To determine whether the
recombinant 148 antibody synthesized in SP2/0 cells
is pentameric, purified 148 antibody was passed over
25 an HPLC gel filtration column (Dupont Zorbax GF
450), and the elution profile compared to that of a
standard human IgM pentameric antibody. The major
148 peak eluted at the same position as the standard
(HA-1A) IgM indicating that the recombinant antibody
30 is of a similar size to the standard.

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HA-1A antibody binds to the lipid A portion of
the lipopolysaccharide (LPS) molecule derived from
gram negative bacteria. To ascertain whether the
148 recombinant antibody retains the binding
05 characteristics of HA-1A antibody, an immunoassay
was performed using purified HA-1A from the original
hybridoma, C83 (the C83 antibodies) and the 148
antibodies. Figure 3 shows the results of the lipid
A binding assay. The binding curves for the two
10 antibodies are indistinguishable, demonstrating that
the 148 antibody (white circles) is equivalent to
the C83 antibody (white squares) in the lipid A
binding assay.

Characteristics of Cell Line 148

15 Figure 4 shows the results of an experiment in
which the C83 cell line was compared to the 148
clone with regard to antibody production and cell
number. The experiment was performed under growth
conditions previously determined to be optimal for
20 C83 (i.e., Iscoves's medium supplemented with 10%
FBS). The amount of antibody produced by 148 and
C83 are comparable (Figure 4A) even though 148 cells
grow to a lower density under conditions optimized
for C83 growth (Figure 4B). These results show that
25 the 148 antibody production per cell is signifi-
cantly higher than that of C83. At the 5 day point,
when cell number is maximum for both cell lines,
clone 148 produces 23 μg/ml/10^6 cells whereas C83
produces 6.8 μg/ml/10^6 cells. As higher 148 cell
30 densities are achieved, production of the

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recombinant antibody would also be expected to in-
crease.

        To assess the stability of the antibody-produc-
ing phenotype for clone 148, the cells were cultured
05 in the absence of the selective agents used during
the initial selection after transfection. Antibody
production decreased approximately 50% over the
course of 60 days in the absence of selection.
Subclones of clone 148 were obtained from the
10 cultures that had been grown for 20 passages
(approximately 60 days) without selection. A
subclone, 148-35, exhibited growth and antibody
production characteristics similar to 148 but is
routinely cultured in the absence of selective
15 agents. These data indicate that cell lines
expressing transfected antibody genes can be ob-
tained that do not require continued selective
pressure to maintain high levels of antibody
production.

20 DISCUSSION

        The present method demonstrates the feasibility
of stabilizing and improving expression of human
monoclonal antibodies by cloning the antibody genes,
e.g., from a hybridoma, and expressing them in a new
25 environment suitable for long term expression. In
the embodiment illustrated in the Exemplification, a
mouse-human heterohybridoma, HA-1A (C83), was used
as the source of human antibody genes. The heavy
and light chain antibody genes were cloned using
30 human J region probes to screen genomic libraries

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made from HA-1A. The HA-1A light chain variable
region was assembled with a previously cloned human
kappa constant region and the entire HA-1A heavy
chain gene into expression vectors containing
05 selectable marker genes for mammalian cells.
Although in this case the entire heavy chain gene
was cloned, it is possible to use just the heavy
chain variable region to assemble with a previously
cloned constant region of the desired isotype to
10 yield an antibody with the desired characteristics.
Appropriate selection of the isotype can be useful
to maximize the therapeutic effectiveness of the
antibody. For example, the human IgG_1 isotype has
been shown to be the most effective human isotype in
15 mediating ADCC killing of human tumor cells. A
cancer therapy monoclonal antibody, therefore, might
be more effective when in the form of an IgG_1.

The transcriptional units in the expression
plasmids utilized the natural human regulatory
20 signals such as promotors, enhancers, and poly-
adenylation sequences. After transfection of the
expression plasmids into the mouse myeloma cell line
SP2/0, a cell line was isolated, designated 148,
that secreted human IgM as determined by ELISA
25 assay.

The antibody secreted by clone 148 appears to
be equivalent to the HA-1A antibody secreted by the
C83 hybridoma. The deduced amino acid sequences
from the cloned DNA are clearly human in character
30 when compared to other mouse and human antibody
sequences. The first 95 amino acids of the light

-24-

chain are identical to the sequence of a human IgM,K
rheumatoid factor, although a different J exon is
utilized. F. Goni et al., J. Immunol.,
135:4073-4079 (1983). Light chains of this subgroup
05 (V$_K$IIIb) comprise approximately 15% of normal Ig.
D.K. Ledford et al., J. Immunol., 131:1322-1325
(1983). A partial N-terminal amino acid sequence
has been performed on the HA-1A antibody and the
first 20 residues are identical to the sequence
10 encoded in the cloned light chain DNA.

Purified recombinant 148 antibody reacted with
an anti-human IgM antibody in an ELISA assay, and
was indistinguishable from the original HA-1A
antibody in immunoreactivity towards lipid A. The
15 recombinant antibody appears to be the correct size
for pentameric IgM as determined by HPLC gel
filtration chromatography.

The successful synthesis of a human antibody in
a murine cell line demonstrates that there is no
20 inherent species-specific barrier to efficient
expression of human immunoglobulin promotors and
enhancers can function in murine cells. Conversely,
murine SP2/0 cells contain all the necessary factors
in addition to synthetic and secretory apparatus to
25 support the expression of exogenously supplied human
antibody genes. Although this cross-species
compatability might have been predicted, a high
degree of sequence conservation between the
immunoglobulin control regions of different species
30 does not guarantee functional equivalence. For
example, the rabbit kappa enhancer region is

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non-functional in mouse myeloma cells despite
extensive sequence homology.

    The 148 cell line that secretes the recombinant
antibody has characteristics desirable for large
05 scale antibody production. Overall IgM secretion
levels are comparable to the original hybridoma and
in fact exceed those of the hybridoma on a per cell
basis. Although 148 antibody secretion gradually
decreases in the absence of selective pressure, a
10 subclone of 148 was obtained which produces high
levels of antibody in the absence of selection for
at least 60 days suggesting that large scale long
term production can be achieved without the addition
of expensive selective agents. The 148 cell line
15 has also been adapted to growth in 10-fold lower
fetal bovine serum than the original hybridoma while
still maintaining high antibody secretion. The
properties of this cell line demonstrate that the
problems of low secretion and loss of production in
20 the absence of selection often seen with expression
of transfected antibody genes can be overcome.

    The characteristics of the 148 cell line show
that human antibody genes can be rescued from
"problem" hybridomas and expressed in a format
25 suitable for high level long-term economical anti-
body production. Cloning of the genes may be
successful even in cases where they are present in
amounts fewer than one copy per cell, e.g., if human
chromosomes are being lost soon after fusion. To
30 rescue these genes, either more plaques could be
screened, or the human variable region sequences

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could be amplified via polymerase chain reaction
prior to cloning. The recipient cell line can be
chosen for its desirable characteristics, for
example favorable growth kinetics, type of growth
05 medium, and lack of endogenous pathogens or other
undesirable contaminants. While avoiding the
problems of low production and instability commonly
associated with human-derived hybridomas, this
approach also offers the advantage of a rational
10 choice of monoclonal antibody isotype to maximize
the effectiveness of a therapeutic antibody.

Equivalents

Those skilled in the art will recognize, or be
able to ascertain by no more than routine experi-
15 mentation, many equivalents of the specific embodi-
ments of the invention described herein. Such
equivalents are intended to be encompassed by the
following claims.

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CLAIMS

1. A method for producing a selected human mono-
clonal antibody, comprising the steps of:

(a) preparing a first nucleic acid sequence

05   encoding a all or a portion of a human
heavy chain of a human monoclonal antibody
and a human regulatory sequence, and a
second nucleic acid sequence encoding a
all or a portion of a human light chain of

10   the selected human monoclonal antibody and
a human regulatory sequence;

(b) transforming a mammalian host cell with
said first and second nucleic acid
sequences;

15 (c) culturing said transformed host cell; and

(d) recovering monoclonal antibody produced by
said cultured host cell.

2. A method of Claim 1 wherein the first and
second nucleic acid sequences are both derived

20 from a hybridoma which produces the selected
human monoclonal antibody.

3. A method of Claim 2 wherein the first and
second nucleic acid sequences are derived from
a human/human or human/murine hybridoma.

25 4. A method of Claim 2 wherein the hybridoma is a
mouse/human hybridoma HA-1A.

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5. A method of Claim 1 wherein the first and
    second nucleic acid sequences are derived from
    different human-antibody-producing hybridomas.

6. A method of Claim 1 wherein the first nucleic
05 acid sequence encodes a variable region of the
    heavy chain of the monoclonal antibody, and the
    second nucleic acid sequence encodes a variable
    region of the light chain of the monoclonal
    antibody.

10 7. A method of Claim 1 wherein the first nucleic
    acid sequence encodes an intact region of the
    heavy chain of the monoclonal antibody, and the
    second nucleic acid sequence encodes an intact
    light chain of the monoclonal antibody.

15 8. A method of Claim 1 wherein the mammalian host
    cell comprises a murine myeloma cell.

9. A method of Claim 8 wherein the murine myeloma
    cell comprises SP2/0-Ag14 or P3X63-Ag8,653.

10. A method of Claim 1 wherein the human mono-
20 clonal antibody comprises an anti-lipid A
    immunoglobulin M antibody.

11. A method for producing recombinant human
    monoclonal antibodies comprising the steps of:
    (a) preparing a first nucleic acid sequence
25   encoding a variable region of a human

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heavy chain of the antibody and a human
regulatory sequence, and a second nucleic
acid sequence encoding a variable region
of a human light chain of the antibody and
05   a human regulatory sequence;

(b)   inserting the first and second nucleotide
       sequences into at least one expression
       vector;

(c)   transfecting a mammalian host cell with
10   the expression vector thereby transforming
       the host cells;

(d)   selectively growing the transformed host
       cells; and

(e)   recovering human monoclonal antibodies
15   produced by the transformed host cells.

12.   A method of Claim 11 wherein the first and
       second nucleic acid sequences are derived from
       a hybridoma which produces the human monoclonal
       antibody.

20 13.   A method of Claim 12 wherein the hybridoma is a
       human/human or a human/murine hybridoma.

14.   A method of Claim 13 wherein the hybridoma is a
       murine/human hybridoma comprising HA-1A.

15.   A method of Claim 11 wherein the first nucleo-
25   tide sequence encodes an intact human heavy
       chain of the antibody.

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16. A method of Claim 11 wherein the second nucleo-
     tide sequence encodes an intact human light
     chain of the antibody.

17. A method of Claim 11 wherein the expression
05   vector is a plasmid.

18. A method of Claim 17 wherein the plasmid vector
     contains a nucleotide sequence encoding a human
     constant region of a human light chain of the
     antibody.

10 19. A method of Claim 17 wherein the plasmid vector
     contains a nucleotide sequence encoding a
     constant region of a human heavy chain of the
     antibody.

20. A method of Claim 17 wherein the plasmid vector
15   contains a selectable marker gene.

21. A method of Claim 17 wherein the selectable
     marker gene comprises gpt or neo.

22. A method of Claim 17 wherein the plasmid
     comprises pSV2gptC83DP.

20 23. A method of Claim 17 wherein the plasmid
     comprises pSV2neoC83DP.

24. A method of Claim 11 wherein the host cell
     comprises a murine myeloma cell.

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25. A method of Claim 24 wherein the murine myeloma
    cell comprises SP2/0-Ag14 or P3X63-Ag8, 653.

26. A method of Claim 11 wherein the human mono-
    clonal antibody comprises an anti-lipid A
05 immunoglobulin M antibody.

27. A murine myeloma cell transformed with the
    plasmid pSV2gptC83DP.

28. A murine myeloma cell transformed with the
    plasmid pSV2neoC83DP.

10 29. Cell line C83-148-3F3-14-21-31.

30. A recombinant human monoclonal antibody pro-
    duced by the cell line of Claim 29.

31. A murine myeloma cell which expresses a human
    monoclonal antibody specific for lipid A of a
15 gram negative bacterial endotoxin.
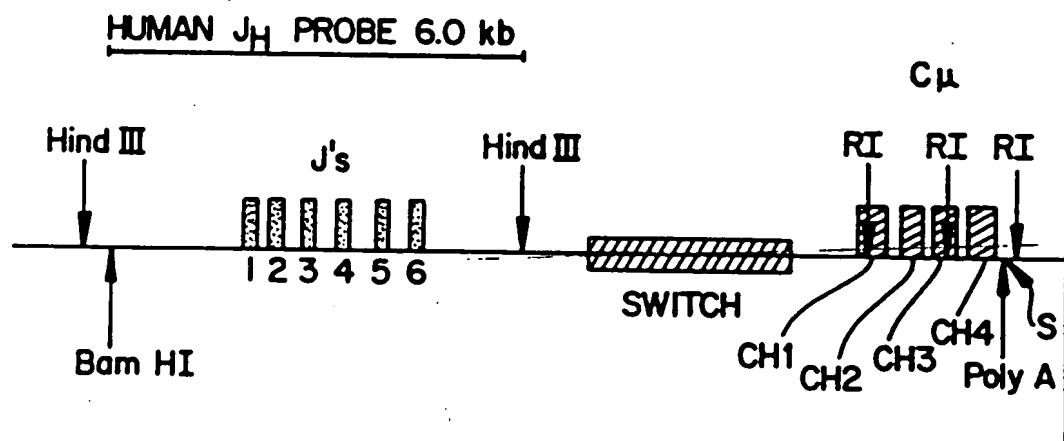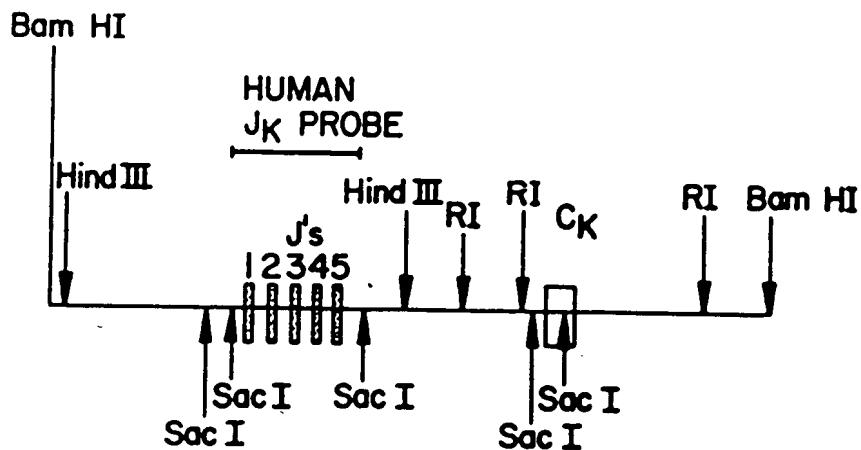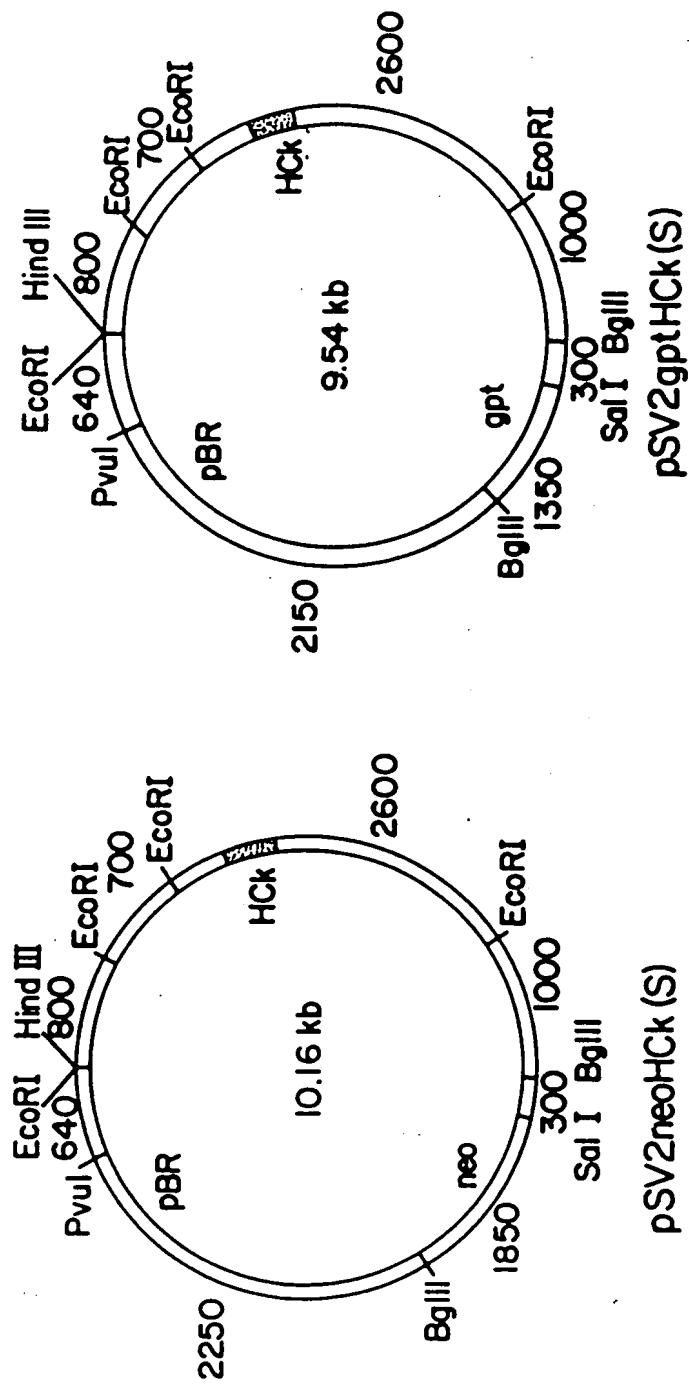
Bam HI

HUMAN
J_K PROBE

Hind III

Hind III RI   RI C_K   RI Bam HI

J's
1 2 3 4 5

Sac I   Sac I

Sac I

Sac I Sac I

Sac I

HUMAN J_H PROBE 6.0 kb

Cμ

Hind III   J's   Hind III   RI   RI   RI

1 2 3 4 5 6

SWITCH

Bam HI

CH1   CH3   CH4   S
    CH2   Poly A

FIG. 1

FIG. 2

Recombinant C83 Immunoactivity
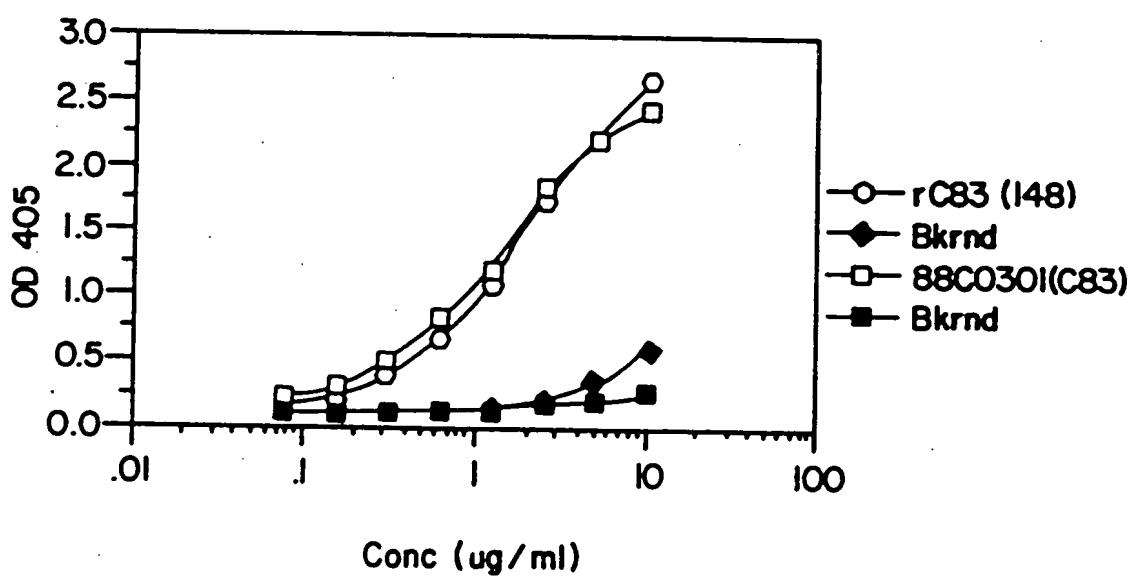
FIG. 3
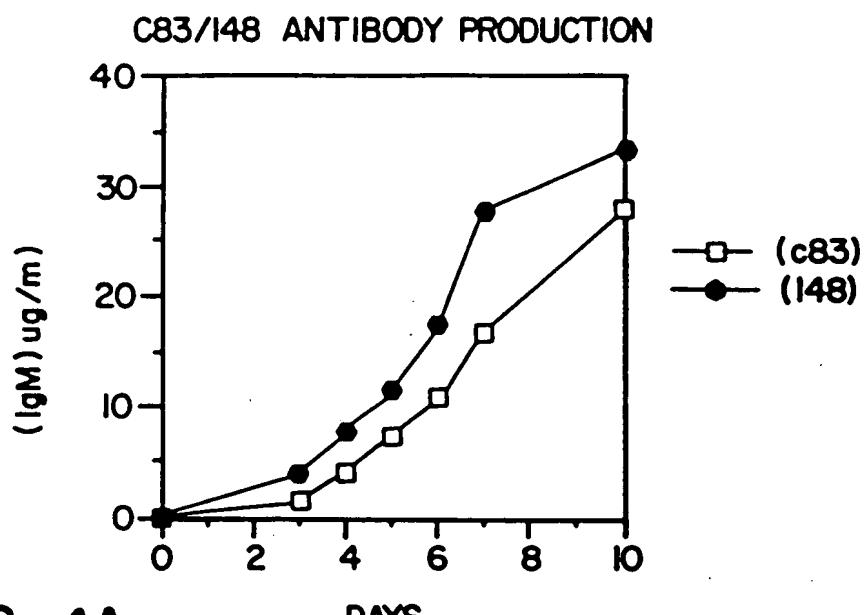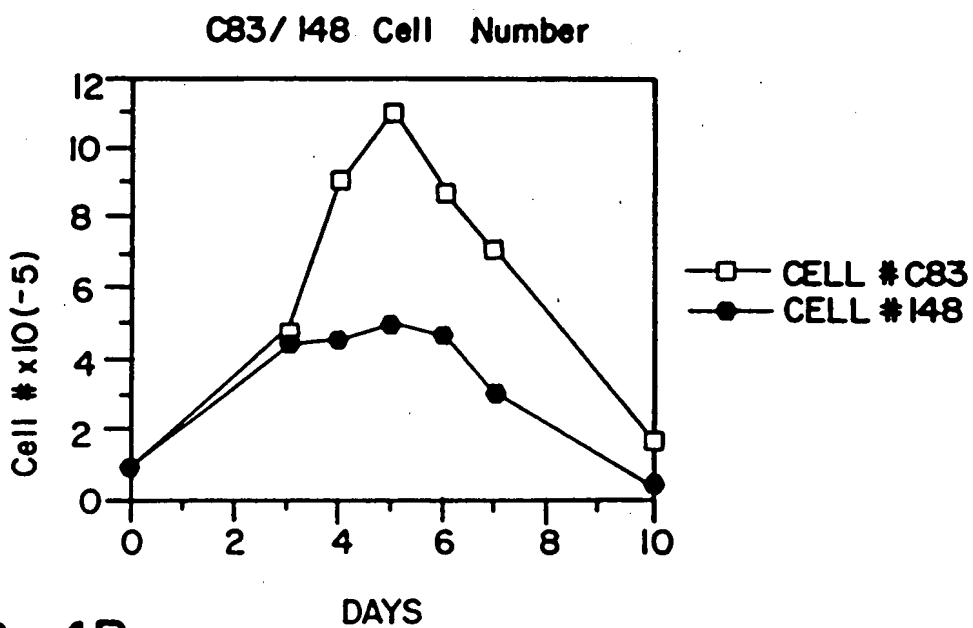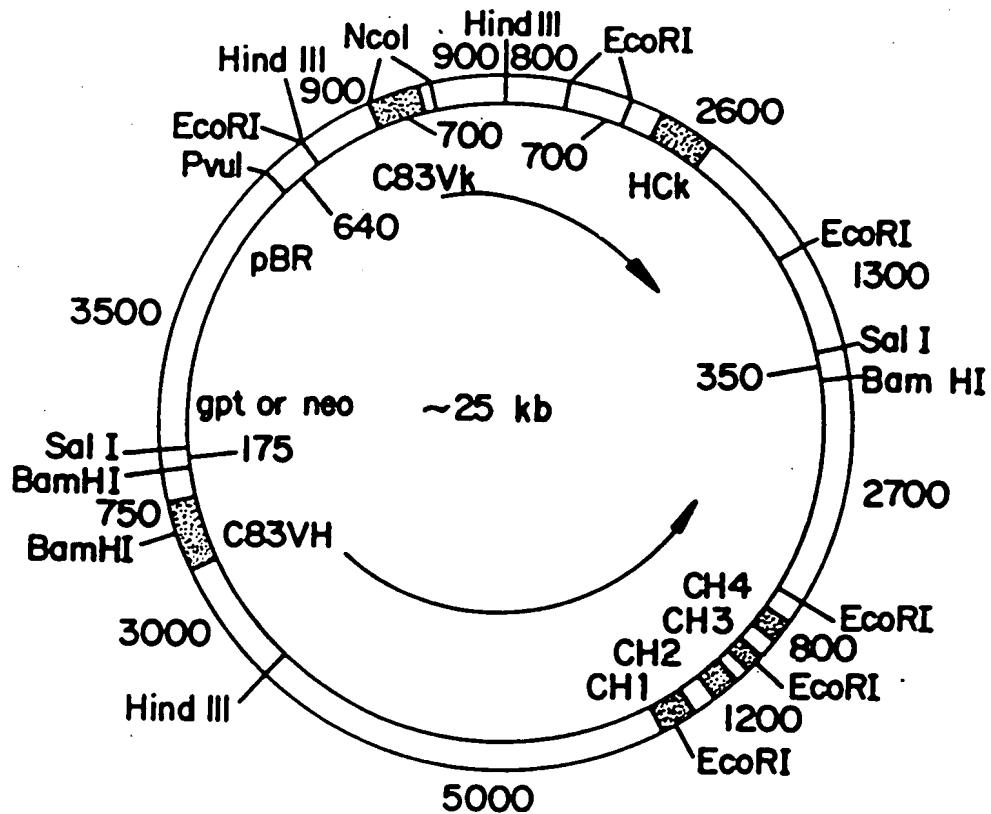
C83/I48 ANTIBODY PRODUCTION

FIG. 4A

C83/ I48 Cell   Number

FIG. 4B

FIG. 5

INTERNATIONAL SEARCH REPORT

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)⁶

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 12 P 21/08, C 12 N 15/13, A 61 K 39/395

II. FIELDS SEARCHED

Minimum Documentation Searched⁷

Classification System	Classification Symbols
IPC5	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in Fields Searched⁸

III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹

Category *	Citation of Document,¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No.¹³
X	EP, A1, 0328404 (MEDICAL RESEARCH COUNCIL) 16 August 1989, see page 4, line 35 - line 44; page 8, line 37 - line 38 --	1,2,5-8, 11-13, 15-19, 24
X	Science, Vol. 229, September 1985, S. L. Morrison: "Transfectomas Provide Novel Chimeric Antibodies ", see page 1202 - page 1207 see page 1206, second column, second paragraph and page 1204 --	1-3,5-8, 11-13, 15-19, 24
Y	EP, A1, 0125023 (GENENTECH, INC. ET AL.) 14 November 1984, see page 18, line 15; page 27, line 13 --	1-3,5- 13,15- 21,24, 26

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the International filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
20th December 1990	1 1. 01. 91

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	Alfredo Prein

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	EP, A3, 0173494 (THE BOARD OF TRUSTEES OF THELELAND STANFORD JUNIORUNIVERSITY) 5 March 1986, see page 10, line 15 - line 20; page 11, line 33; page 12, line 12; page 20	1-3,5-13,15-21,24,25
A	Nature, Vol. 312, December 1984, G. L. Boulianne et al.: "Production of functional chimaeric mouse/human antibody ", see page 643 - page 646	1-3,5-13,15-21,24,25
A	Nature, Vol. 312, December 1984, M. S. Neuberger et al.: "Recombinant antibodies possessing novel effector functions ", see page 604 - page 608 see page 605, right column and fig 1	1-3,5-13,15-21,24,25
A	EP, A2, 0315062 (BRISTOL-MYERS COMPANY) 10 May 1989, see page 6-8 and claims	1-3,5-13,15-21
A	Science, Vol. 229, August 1985, Jean L. Marx: "Antibodies Made to Order ", see page 455 - page 456 see page 455, bridging paragraph between second and third column	1-3,5-13,15-21
A	EP, A1, 0314161 (BRISTOL-MYERS COMPANY) 3 May 1989, see page 3, line 20; page 16, line 23 see claims	1-3,5-13,15-21
P,X	EP, A2, 0388964 (BOEHRINGER MANNHEIM GMBH) 26 September 1990, see page 3, lines 16-29 and 32	1,11

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	National Library of Medicine, File Med 88, Accession no. 88297774, Ramachandra RN: "Human--Human hybridomas secreting lipid A reactive monoclonal antibodies", Immunol Lett 1988 Jun;18(2):93-7	31
Y		26
	--	
X	EP, A1, 0271379 (ROUSSEL-UCLAF) 15 June 1988, see claims	31
Y		26
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ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/US 90/05322

SA   40359

This annex lists the patent family members relating to the patent documents cited in the above-mentioned International search report.
The members are as contained in the European Patent Office EDP file on 28/11/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		GB-A-	2216126	04/10/89
		WO-A-	89/07452	24/08/89
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		AU-D-	2642984	11/10/84
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		CA-A-	1218613	03/03/87
		JP-A-	60155132	15/08/85
		US-A-	4816567	28/03/89
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		JP-A-	61119196	06/06/86
EP-A2- 0315062	10/05/89	AU-D-	2444688	27/04/89
		JP-A-	2000477	05/01/90
EP-A1- 0314161	03/05/89	AU-D-	2436388	01/06/89
		JP-A-	2138989	28/05/90
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EP-A1- 0271379	15/06/88	FR-A-B-	2606421	13/05/88
		JP-A-	1137993	30/05/89